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## 13. ABSTRACT (Maximum 200 words)

The spore coat of Bacillus subtilis is comprised of two morphological layers each consisting of several polypeptides. One which was cloned and sequenced is processed from a precursor. A deletion of this gene resulted in some alteration in the structure of the inner coat and of the capacity to respond to a specific spore germinant. When this protein was overproduced from a multicopy plasmid, the spores had a thickened inner coat and germinated poorly. The absence of another spore coat protein had extensive pleiotropic effects on the assembly of the outer spore coat. As a result, the spores were lysozyme-sensitive but they germinated at rates similar to the wild type. Antibody was used to demonstrate that this particular coat protein was synthesized long before the assembly of the spore coat and much earlier than other coat proteins. Despite its hydrophilic properties, it is located in the cell insoluble (membrane) fraction requiring detergent or 8M urea for solubilization. Suppressor strains forming lysozyme-resistant spores were selected from those produced by this deletion strain. The germination properties of these spores were unaltered but novel polypeptides were present in spore coat extracts.

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## A. Statement of the Problem Studied

The coat of bacterial spores usually consists of two morphological layers which function to protect spores from various solvents and enzymes such as lysozyme. It may also have a direct or indirect role in germination. Little is known, however, about the assembly of the coat, the properties of the polypeptides present in each layer, and the function(s) of each in solvent resistance, germination or assembly. Among the spore formers, Bacillus subtilis has been selected for the study of these problems because of the genetic, biochemical and cloning techniques available. A variety of approaches centering on genetic engineering technology have been used to define the structure and function of particular B. subtilis spore coat proteins.

## B. Summary

1. Both the precursor and mature CotT proteins were partially sequenced in order to confirm that a 10.1 kDa precursor initiated with a TTG codon and was processed at an arg-gln bond to produce the 7.8 kDa mature protein. Site directed mutagenesis was used to change the arg to ile (confirmed by sequencing). A heterozygous diploid was constructed and the spores were very similar to the wild type in protein profile and germination responses. Extracts of this diploid contained an excess of CotT precursor detectable in immunoblots whereas little if any was found in the parental strain or in a diploid with two copies of the wild type cotT gene. This modified CotT protein appears not to be processed but its effect in single copy must be determined. Strains containing only a copy of the mutant gene were isolated as chloramphenicol-sensitive revertants of the diploid. The presence of only one copy of the cotT gene was confirmed by Southern hybridization. These revertants accumulated CotT precursor in extracts (Fig. 1) or on spores.

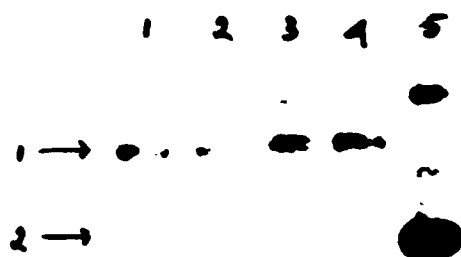


Fig. 1. Immunoblot with CotT antibody of spore extracts from *B. subtilis* JH642 mutants containing a single copy of the cotT gene altered at the processing site (lanes 1-4) and from *B. subtilis* JH642 (lane 5). Arrow 1 indicates precursor CotT antigen present in extracts of spores produced by the mutants and arrow 2 shows the processed CotT antigen in extracts of spores from the parental strain.

2. As previously mentioned, the CotT protein is over expressed in a gerE strain with most of the precursor in extracts of sporulating cells (Aronson et al. in publication list). Two deletion mutants of cotT were constructed and one contained an intact gene with the presumed RNA polymerase binding site (consistent with nuclease  $S_1$  mapping) but lacked the upstream region. No CotT antigen was produced by this strain and the spores had the same germination defect as a deletion which contained two defective gene copies. Apparently, an upstream region (perhaps the GerE protein binding site) is needed for transcription. We have sent the cotT clone to Dr. R. Losick at Harvard for a further study of regulation (cotT-lacZ fusions). In return, he has sent clones of the cotD and cotE genes (as well as deletion strains) for our analysis of spore properties and coat assembly. We are in communication to be certain that different coat genes are being cloned and studied so that our results will complement each other.

3. The cotT deletion strains germinated poorly in the presence of the glucose-fructose-asparagine mixture but the same as the wild type in L-alanine (plus inosine) or Penassay broth (Bourne et al. in publication list). The slow germination rate was unchanged when the concentration of germinants was decreased ten fold or increased two fold. Perhaps, the absence of the CotT protein alters the coat structure subtly (little difference from the wild type seen in electron micrograph sections) and thus access to a target for this particular germination mixture.

Increasing the copy number of the cotT gene on a low copy number plasmid resulted in spores which germinated slowly in response to each of three germination systems. These spores have extra inner coat layers and a large amount of precursor antigen was extracted from well washed spores. In fact, there appears to be two CotT precursor proteins present, a major one of 10.1 kDa and a second one of about 8.5-9.0 kDa. Since these precursors can associate tightly with spores, they may be processed after deposition.

4. The cotT gene has been subcloned into an T<sub>7</sub> polymerase expression vector kindly provided by Dr. W. Studier and a 10 kDa band was detected in cell extracts by staining and immunoblotting. The processing protease (probably trypsin-like) is of interest especially since it may be a spore specific protease with other functions related to coat morphogenesis. In vitro, trypsin but not the B. subtilis serine proteases appeared to cleave the CotT precursor to a polypeptide of the correct size (Aronson et al.). A B. subtilis mutant lacking an intracellular trypsin-like activity still processed CotT precursor so a special protease(s) may be involved.

5. A limited number of studies have been done with the cotD gene and its deletion clone kindly provided by Dr. Losick. CotD overproduction (stained band on SDS-PAGE of spore or cell extracts) resulted in some decrease in

germination for all three germinants but not as marked an effect as overproduction of the CotT protein (on the same low copy number plasmid so gene copy number should be about equal; see Bourne et al.). There were no detectable germination effects due to a deletion of the cotD gene.

6. There was some overproduction of the CotE protein when cloned on a low copy number vector but no noticeable effect on germination nor change in the lysozyme-sensitivity of the spores. Apparently, extensive overproduction of this protein inhibits sporulation (B. Zheng and K. Losick, personal communication) but some overproduction in the low copy number plasmid was evident from stained SDS-PAGE. Since the protein is difficult to detect in stained gels, an antibody was produced by synthesizing a peptide of 20 residues from the sequence at the carboxyl end. This peptide was purified on HPLC and coupled to bovine serum albumen for subcutaneous injection into rabbits. The antibody is specific for the CotE protein and was used in immunoblots to confirm overproduction in the strain containing the clone as well as the absence of CotE antigen in the deletion strain.

CotE antigen appeared early in sporulation (Stage II-III) and was not present in a spoOB mutant. Once produced, the CotE protein was stable since it survived prolonged incubation of sporulating cells in chloramphenicol. Even though it appears to be a hydrophilic protein, CotE antigen was found primarily in the crude insoluble fraction after lysis by sonication (Fig. 2).

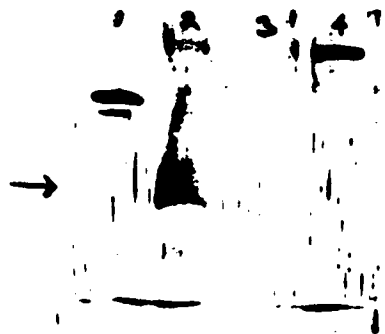


Fig. 2. Immunoblot with CotE antibody of extracts from sporulating cells (stage II-III, i.e. before the appearance of phase-white endospores) of the wild type, PY17, (lanes 1 and 2) and the cot E deletion strain (lanes 3 and 4). Lanes 1 and 3 contain soluble fractions prepared by sonication and centrifugation at 20,000 xg for 10 min. Lanes 2 and 4 contain 6M urea--1% SDS pH 9.5 extracts of the insoluble fractions. Arrow on left indicates a broad band of CotT antigen of ca. 12kD. Note also smaller reacting antigen which may be a degradation product. Sharp reacting band of higher molecular weight in lanes 1 and 4 is present usually in the soluble fraction of all cells (even during exponential growth and in a spoOB mutant).

Endospores were separated from this crude membrane fraction in a Renografin gradient and both fractions contained CotE antigen whereas CotT antigen was primarily present in the spore extract. The protein was not solubilized with low or high (0.5M KCL) ionic strength buffer, with 1% Triton X-100, Brij 35 or n-octyl-glucoside. Either SDS or 8M urea was needed for solubilization.

A data bank search revealed some homology over a stretch of ca. 65 amino acids in the middle of the protein to an acidic cytokeratin intermediate filament protein from bovine bladder urothelium. There are 17 identical and 29

similar residues within this region which is believed to form an a helix in the cytokeratin important for the protein interactions required for filament formation. The homology is intriguing because (1) intermediate filament proteins have not been found in prokaryotes and because (2) one of the phenotypic effects of a cotE deletion is the production of very small condensed germinated spores (see below) perhaps due to the absence of some critical spore structural component.

Germination studies have been done with the cotE deletion and there was a short lag (5-10 minutes) in L-alanine (plus inosine and KCL; Fig. 3),

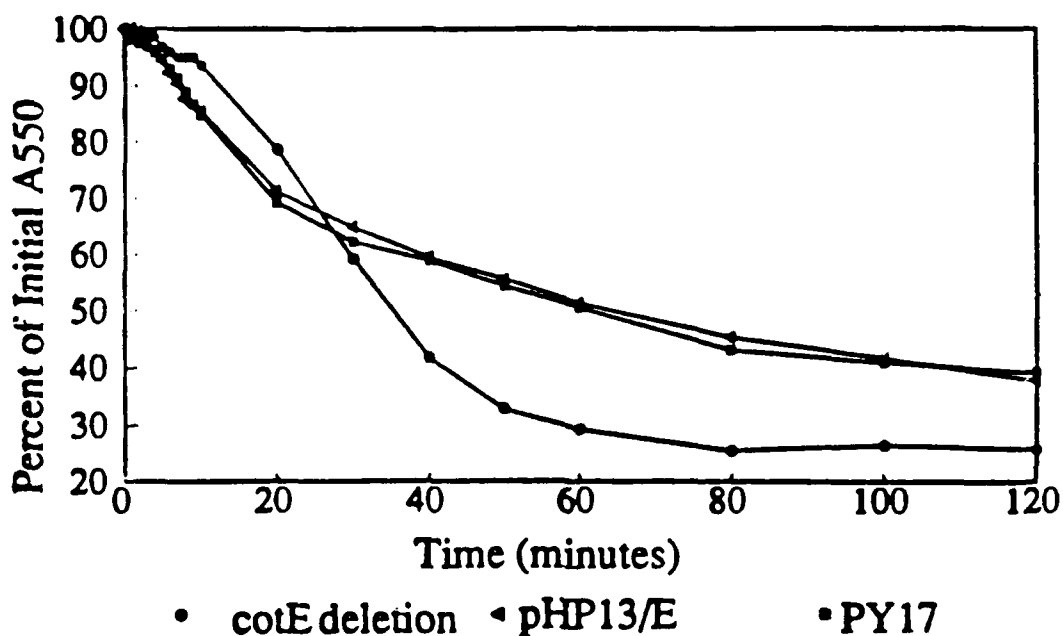


Fig. 3. Germination of spores of the wild type (PY17), the cotE deletion and a strain with the cotE gene on a low copy number plasmid (pHP13/E). Spores were suspended to an A<sub>550</sub> value of 1.0, and heat activated at 80°C for 20 min. Germination was in 0.05M Tris-0.1M KCL, pH 7.6 plus 1mM each of L-alanine and inosine.



but not in Penassay broth. The lag was found with or without heat activation and even when the L-alanine concentration was increased tenfold (to 10mM) but not if KCL were omitted from the germination mixture. Germination proceeded after the lag perhaps more rapidly than in the control and always to a much lower optical density (i.e. 80% decrease versus about 50-60% in the control; Fig. 3). There was little lysis of these germinated spores as determined by viability assays before and after germination for 120 min. The germinated cotE spores were very condensed in appearance in the phase microscope which could account for the extensive decrease in light scattering. There are obviously a lot of interesting properties of this cotE deletion strain in relation to coat structure, assembly and germination. CotE antibody was sent to Dr. Losick for electron microscope studies of the localization of this antigen.

Lysozyme-resistant spores produced by the cotE deletion strain were isolated from B. subtilis Marburg containing the deletion. The presence of the deletion was confirmed by transforming the marker (chloramphenicol<sup>R</sup>) to B. subtilis JH642 and by Southern hybridization. Two suppressor strains have been studied and both were as solvent-resistant (n-octanol, chloroform) and the spores germinated the same as the original deletion strain. There were one or two novel polypeptides of ca. 25 kDa in extracts of spores or spore coats from the suppressor strains but most of the other high molecular weight outer spore coat proteins were still absent. Both suppressors appear to be linked to the aroD gene but more extensive mapping is needed.

One other interesting property of the spores formed in the cotE deletion strain is the presence of CotT antigen primarily in the precursor form (Fig. 4).

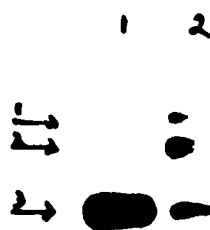


Fig. 4. Immunoblot with CotT antibody of spore extracts from the wild type strain PY17 (lane 1) and the CotE deletion strain (lane 2). The processed CotT antigen is prevalent in the wild type (arrow 3) whereas the precursor forms of CotT (arrows 1 and 2) are present in spores lacking outer coat due to the cotE deletion.

This protein is probably present in the inner coat but apparently is not processed in the absence of outer coat. A possibly functional relation between processing and outer coat assembly will be studied in the strain with the Cot1 processing mutation.

Two other coat genes are being cloned. In one case, a coat polypeptide was transferred to a PVDF membrane for sequencing. In the other, the coat insoluble fraction (i.e. 20-30% of the protein remaining after extensive extraction of purified spore coats) was digested with formic acid and the peptides were resolved on HPLC. One pure peptide was sequenced and mixed probes prepared. Southern transfers of restriction enzyme digests of B. subtilis JH642 DNA were hybridized with these probes. Some HindIII fragments have been subcloned and will be sequenced using the probes as primers.

### C. PUBLICATIONS

- Aronson, A. I., Song, H-Y. and N. Bourne (1989) Gene structure and precursor processing of a novel Bacillus subtilis spore coat protein. Molec. Microbiol. 3:437-444.
- Bourne, N., Huang, T-S. and A. I. Aronson (1990) Properties of Bacillus subtilis spores with alteration in spore coat structure. In Genetics and Biotechnology of Bacilli. A. T. Ganesan and J. A. Hoch (eds.) in press.
- Bourne, N., FitzJames, P. C. and A. I. Aronson. Germination defects in Bacillus subtilis spores altered in spore coat protein composition. J. Bacteriol., under revision.

#### Abstract

Bourne, N. and A. I. Aronson. Properties of Bacillus subtilis spores with alterations in spore coat structure. Fifth International Conference on Genetics and Biotechnology of Bacilli. July 9-12, 1989, Pacific Grove, California.

### D. Participating Scientific Personnel

Dr. N. Bourne, Postdoctoral Associate	1987-1989
Mr. J. Zhang, Graduate Assistant	1988-present
Dr. L. Ekanayake, Postdoctoral Associate (part time)	1990

# Gene structure and precursor processing of a novel *Bacillus subtilis* spore coat protein

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## Summary

The gene for an unusual 8kD *Bacillus subtilis* spore coat polypeptide has been cloned and sequenced. It contains high percentages of proline, glycine and tyrosine, lacks thirteen amino acids, and is present as the carboxyl two-thirds of an open reading frame encoding a 12kD polypeptide. Two presumptive precursors which could be converted to the 8kD antigen by incubation with trypsin were found in extracts of cells or spores of a strain containing multiple copies of this gene. Large amounts of these coat antigens were also present in extracts of a germination-defective mutant which is altered in spore coat structure. There was little 8kD coat protein in the mutant, however, implying that processing is dependent on proper coat assembly. This gene was mapped to the *metA* region of the *B. subtilis* chromosome, a unique location as is true for other spore coat genes. Transcription and translation occurred late in sporulation (stage V) and the upstream region contained sequences similar to those found in other spore coat genes.

## Introduction

The proteinaceous coat layers of the bacterial spore (exclusive of the exosporium) are believed to have a protective function and to play some role in germination (Aronson and Fitz-James, 1976; Kutima and Foegeding, 1987). A cryptic cortex lytic enzyme (Brown *et al.*, 1982; Foster and Johnstone, 1987) or proteases involved in the activation of this enzyme (Boschwitz *et al.*, 1985) may be associated with an integral part of the coat. Alternatively, the coat may simply function as a barrier or conduit for germination factors reaching their target at the inner spore membrane (Skomurski *et al.*, 1983).

The nature and heterogeneity of the proteins comprising the spore coat vary from a rather simple array of low molecular weight polypeptides in *Bacillus cereus* (Aronson and Fitz-James, 1976), *Bacillus megaterium* (Stewart

and Ellar, 1982), and perhaps clostridia (Tsuzuki and Ando, 1985) to a rather heterogeneous group of polypeptides in *B. subtilis* (Goldman and Tipper 1978; Pandey and Aronson, 1979; Jenkinson *et al.*, 1981). The appearance in freeze-etched electron micrographs of the surface layers of the latter spores also differs considerably from the others (Holt and Leadbetter, 1969; Aronson and Fitz-James, 1976). In the case of *B. subtilis*, the extractable proteins vary in size from about 65kD to 7kD with a preponderance of 4–5 species of 7–12 kD. In fact, some of the larger, less prevalent proteins found in SDS-PAGE may be contaminants or superficial components since their presence varies with the medium used for spore formation, with the age of the spores and perhaps with the methods used to prepare spore coats or to extract spore coat proteins (Jenkinson *et al.*, 1981; Sastry *et al.*, 1983; Feng and Aronson, 1986).

In addition, about 30% of the protein in *B. subtilis* spore coats is not soluble under a variety of conditions and is probably composed of cross-linked polypeptides, in part antigenically similar to some of those found in the soluble coat fraction (Pandey and Aronson, 1979; Feng and Aronson, 1986). The nature of the cross-linking is not known (Pandey and Aronson, 1979; Goldman and Tipper, 1981).

To date, efforts to isolate mutants defective in spore coat assembly or structure in *B. subtilis* have not been very fruitful. There is only a single germination defective mutant, GerE (Moir *et al.*, 1979; Moir, 1981), altered in spore coat structure. This gene appears to encode a DNA-binding protein (Holland *et al.*, 1987) and this mutant is pleiotropically altered in coat protein composition (Jenkinson and Lord, 1983; Feng and Aronson, 1986). In addition, there are a few other less well-defined presumptive coat mutants (SpoVI, A, B, C) (Jenkinson, 1981; 1983; James and Mandelstam, 1985).

The complexity of *B. subtilis* spore coat polypeptides poses an interesting problem regarding assembly of a protective structure which may be directly involved in germination. As a start in dissecting this complex process, several spore coat genes have been cloned and their regulation as well as their functions have been examined (Donovan *et al.*, 1987; Sandman *et al.*, 1988). Here we report the cloning and analysis of the gene for one of the more prevalent and unusual low molecular weight spore coat proteins (Goldman and Tipper, 1978). It appears that

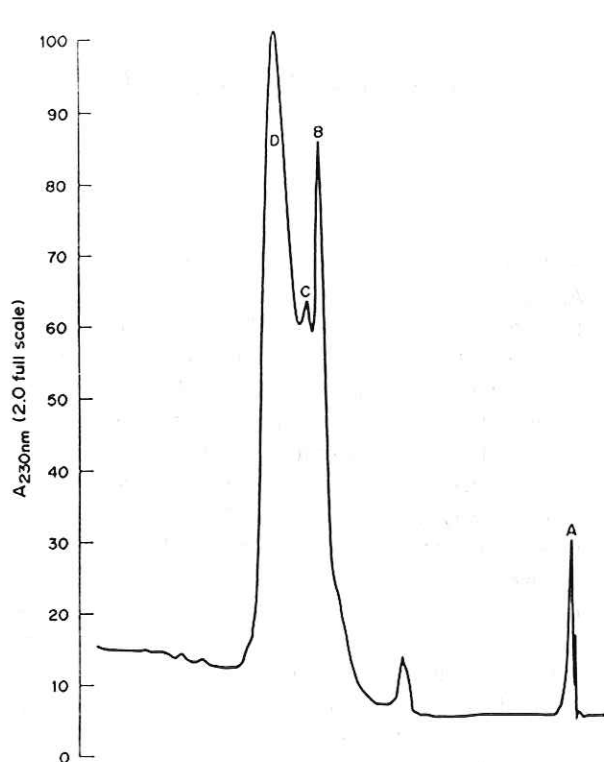


Fig. 1. HPLC elution profile of the  $\text{Na}_2\text{SO}_4$  precipitate of a spore coat extract. The precipitate was dissolved in 20% n-propanol-0.1% TFA and loaded onto an RP-P column. Elution was performed with a linear gradient of 20–100% n-propanol-0.1% TFA. Peaks B–D (eluting at about 40% n-propanol) were collected manually, pooled from several runs, lyophilized and rechromatographed. Initially, peak D was sequenced in order to prepare an oligonucleotide probe. Subsequently 20–30 residues of purified peaks B and C were sequenced and found to be identical to peak D.

the mature coat protein is processed from a precursor, perhaps on the spore surface.

## Results

### Isolation of a spore coat protein and cloning of the gene

One of the major, low molecular weight spore coat proteins was salted out from a spore coat extract with 0.1 M  $\text{Na}_2\text{SO}_4$  (Goldman and Tipper, 1978). Following extensive washing with distilled water, the precipitate was dissolved in 20% n-propanol-0.1% TFA and fractionated on a reversed phase column (Fig. 1). Peaks B–D were collected manually, lyophilized and refractionated in the same system as in Fig. 1. Initially, purified peak D was lyophilized and sequenced:  $\text{NH}_2$  gln-pro-tyr-tyr-tyr-pro-arg-pro-arg-pro-pro-phe-tyr-pro-pro-tyr-tyr-tyr-pro-arg. A mixed deoxyoligonucleotide encoding the underlined amino acids (the pro-tyr-tyr-tyr-pro portion is repeated)

was synthesized:  $\text{CA}^{\text{A}}\text{GCCXUA}^{\text{U}}\text{C}^{\text{U}}\text{UA}^{\text{U}}\text{C}^{\text{U}}\text{UA}^{\text{U}}\text{CCC}$ . It was later found that the sequence of the first twenty amino acids for peaks B and C were the same as for peak D (unpublished results). There is no evidence for multiple genes based on Southern hybridization of the cloned 1.2 kb *Hind*III fragment (see below) to total *B. subtilis* DNA digested with *Hind*III, *Eco*RI or *Bam*HI (unpublished results). It is assumed, therefore, that the three closely eluting peaks in Fig. 1 reflect the same polypeptide bound to some other components, but this possibility has not been explored any further.

The purified oligonucleotide was labelled and hybridized to pools of plasmids containing fragments from a partial *Sau*3A digest or to Southern blots of a *Hind*III digest of *B. subtilis* DNA, as described in the *Experimental procedures*. In both cases, a 1.2 kb *Hind*III fragment was isolated and sequenced (Fig. 2). Only 759 nucleotides are shown, since this region includes the major open reading frame (ORF) encoding a polypeptide of 107 amino acids (13 kD) including the sequence of peak D (the gene encoding this ORF is designated *cotT*). The only other ORFs greater than 40 amino acids long extensively overlapped *cotT*. The sequence of peak D starts about one third of the way from the amino end of the ORF (thick arrow in Fig. 2) and would encode a polypeptide of 7.8 kD. S1 nuclease mapping using either a *Hind*III-*Hha*I end-labelled fragment or primer extension as described in *Experimental procedures* placed the start of transcription as indicated by the thin arrow in Fig. 2. In addition, there are sequences (underlined) in this region which are similar to the presumed RNA polymerase binding sites for other spore coat genes (Zheng *et al.*, 1987, Abstract of the Fourth International Conference on Genetics and Biotechnology of Bacilli) but the closest potential ribosome binding site is 25–27 nucleotides from the ATG codon. Since the optimum spacing is about nine nucleotides (Stormo, 1986), secondary structure is probably important for translation of this mRNA. Data presented below are consistent with the presence of one or more precursors of the 7.8 kD spore coat protein and thus the translation of the larger ORF.

The amino acid composition of the 7.8 kD processed product is very similar to that of the  $\text{Na}_2\text{SO}_4$  precipitate from spore coats and is virtually identical to that of peak D (Table 1). This polypeptide is very unusual because of the absence of thirteen amino acids and the very high content of glycine, tyrosine and proline. There are several repeats of pro-tyr-tyr-tyr-pro or pro-tyr-tyr-pro as well as pro-arg (or pro-arg-pro-arg) and all of the eleven gly residues interspersed with tyr are confined to the carboxyl end.

A single transcript found only in late sporulating cultures hybridized to the 1.2 kb *Hind*III fragment (Fig. 3). The extrapolated size based on ribosomal RNAs and the glutamine synthetase transcript was approximately  $10^5$  Daltons.

tipper STRAND L LIMITS 228 551

750 740 730 720 710 700 690  
ATGAAACCAT TTGATTCITT TTGCCAACAA CAAAACTCC TTCTTTCGTA GCCATTTCGA TTTACTCTTC

680 670 660 650 640 630 620  
ATCAGITGGA ACTGTATCTA TTTAGAAATG TTTTGTAAAC ATTGTAGTAA CATTTATTTC ACATTGTAA

610 600 590 580 570 560 550  
TACTATGAGA TTTAGAAGIT CATGTCAATC CTTTITAAGG AATTTCATCA TAAAAAAAC CGAATTTC

522 492  
ATG AAT GTA CAT ACA CCC AAC TTA AGC ATC AGG AAT ATG GTA AAA GGA ATA AAA AAA GCT  
Met Asn Val His Thr Pro Asn Leu Ser Ile Arg Asn Met Val Lys Gly Ile Lys Lys Ala

462 432  
AGG GAG GTT TTC CTC TTG GAT TAC CCT TTG AAT GAA CAG TCA TTT GAA CAA ATT ACC CCT  
Arg Glu Val Phe Leu Leu Asp Tyr Pro Leu Asn Glu Gln Ser Phe Glu Gln Ile Thr Pro

402 372  
TAT GAT GAA AGA CAG CCT TAT TAT TAT CCG CGT CCG AGA CCG CCA TTT TAT CCG CCT TAT  
Tyr Asp Glu Arg Gln Pro Tyr Tyr Tyr Pro Arg Pro Arg Pro Pro Phe Tyr Pro Pro Tyr

342 312  
TAT TAT CCA AGA CCG TAT TAT CCG TTC TAC CCG TTT TAT CCG CGC CCG CCT TAT TAC TAC  
Tyr Tyr Pro Arg Pro Tyr Tyr Pro Phe Tyr Pro Phe Tyr Pro Arg Pro Pro Tyr Tyr Tyr

282 252  
CCG CGC CCG CGA CCG CCT TAC TAC CCT TGG TAC GGT TAC GGC GGA GGT TAT GGC GGA CGA  
Pro Arg Pro Arg Pro Pro Tyr Tyr Pro Trp Tyr Gly Tyr Gly Tyr Gly Tyr Gly Tyr Gly

220 210 200 190  
TAT GGG GGA GGT TAC GGT TAC TAG TCGCCACA TATCAAAATG GGGCCAGTCC AGCTGCCCAT  
Tyr Gly Gly Gly Tyr Gly Tyr End

180 170 160 150 140 130 120  
TTTTTTGTTT TAAAAATGAC AATATCATGA CAAATATGGG AAATAATTGT GATTCTGTCC CCGTCTTGTT

110 100 90 80 70 60 50  
ACGATAACAA AAGCATGCAT TTTGGGGAGC GTCTATTTTT GGAGAATACA AGAAATCTGT CAGCCATATC

40 30 20 10  
TGAACAAAAA TACATAAAG CTTCATGCC TGCAGGTCGA CTCTAAGGA

**Fig. 2.** Nucleotide sequence of 759 bases of the 1.2 kb *Hind*III fragment and the amino acid sequence of the open reading frame (ORF). The start site of transcription based on S1 nuclease mapping is indicated by a thin arrow. Possible binding sites for RNA polymerase based on comparable sequences in other spore coat genes are underlined. A thick arrow indicates the start of the sequenced spore coat protein and inverted arrows designate a potential termination loop. The sequence used for preparing an oligonucleotide for S1-nuclease mapping is indicated by a broken overline. The location of the *Hha*I site is also marked. These sequence data will appear in the EMBL/GenBank/DBJ Nucleotide Sequence Databases under the accession number X13740.

### Mapping of the *cotT* gene

The *cotT* gene was mapped by integration into the *B. subtilis* chromosome via plasmid pDE194 and selection for *Cm*<sup>R</sup>. The *Cm*<sup>R</sup> marker was mapped by transduction employing the strains of Dedonder *et al.* (1977) and by transformation using the strains listed in *Experimental procedures* (Table 2). The gene mapped to the *metA*, *C*, *D* region of the chromosome but the extent of cotransformation with *metA* was only 9%. This location is distinct from other spore coat genes mapped to date (Donovan *et al.*, 1987), each of which has its unique site (Fig. 4).

### Possible precursor antigens: overproduction and processing

In extracts of late sporulating cells, a possible precursor as well as the 7.8 kD coat antigen were detected (Fig. 5). The larger antigen was about 13 kD in size (based on RNase and cytochrome *c* standards), the size expected from the ORF (Fig. 2). Cells containing multiple copies of pT5A produced much larger quantities of the 13 kD antigen as well as a somewhat smaller 'precursor'. Spore extracts

**Table 1.** Amino acid composition of spore coat protein (mol. %).

	Coat extract <sup>a</sup>	Cloned gene		
		Total ORF	Carboxyl portion <sup>b</sup>	Peak D <sup>c</sup>
Ala	0.96	0.9	0.0	0.0
Arg	13.00	8.3	10.0	10.0
Asn	1.25	3.7	0.0	0.0
Asp				
Cys	0.01	0.0	0.0	0.0
Gln	2.18	2.8	1.8	2.0
Glu		3.7	0.0	0.0
Gly	19.11	11.1	17.8	17.0
His	0.18	0.9	0.0	0.0
Ile	0.45	2.8	0.0	0.0
Leu	0.89	3.7	0.0	0.0
Lys	0.52	2.8	0.0	0.0
Met	0.01	1.9	0.0	0.0
Phe	6.00	4.6	5.0	5.0
Pro	24.20	20.4	30.0	29.0
Ser	0.99	1.9	0.0	0.0
Thr	0.38	1.9	0.0	0.0
Trp	—	0.9	1.8	2.0
Tyr	32.20	22.2	35.0	35.0
Val	0.57	2.8	0.0	0.0

a. From Goldman and Tipper (1978).

b. Start based on protein sequencing (see Fig. 2).

c. See Fig. 1.



Fig. 3. Northern blot (Thomas, 1980) of RNA prepared from *B. subtilis* JH642 during exponential growth (lane 1), when most of the cells had formed phase-white endospores (lane 2), and 1.5 h later (lane 3). Hybridization with nick-translated 1.2 kb *Hind*III fragment (first three lanes) or with an internal fragment from the glutamine synthetase structural gene, *E11* (Strauch *et al.*, 1988) to RNA from exponential cells (lane 4). Marks to the right of lane 4 indicate positions of rRNAs.

contained large amounts of the 7.8kD antigen and surprisingly, the two 'precursor' antigens were present on spores from the strain containing multiple copies of pT5A.

These larger antigens were not removed by extensive washing of the spores (repeating the protocol in *Experimental procedures*) and resulted in spores which responded slowly (3–5 times) to the germinants L-alanine plus adenosine or Penassay broth (unpublished results). Pretreatment of the antibody with HPLC purified spore coat protein (peak D in Fig. 1) coupled to Sepharose 4B resulted in no reaction with all three antigenic species (Fig. 6, lane 8).

It is known that a particular germination mutant, *GerE* (Moir, 1981) has pleiotropic alterations of the spore coat, including the absence of this 7.8kD spore coat protein (Jenkinson and Lord, 1983; Feng and Aronson, 1986). The cloned gene on plasmid pT5A was introduced into a *gerE* strain, AA9-1, but the spores still germinated slowly and were lysozyme-sensitive. Surprisingly, the *gerE* strain overproduced *cotT* precursor antigen even without extra gene copies (Fig. 6). Despite the excess precursor, there was less 7.8kD antigen in cell or spore extracts of the *gerE* strain than in the wild type.

On the basis of the *cotT* ORF, the arg-gln sequence is the apparent site of cleavage for producing the 7.8kD spore coat protein (see the thick arrow in Fig. 2). This peptide bond should be susceptible to trypsin, so cell and spore extracts were dialysed against 0.02 M Tris-HCl, pH 8.0 and incubated with trypsin (Worthington, TRL-3 at 1:50). There was a decrease in the presumed precursor antigens and an increase in one of 7.8kD (Fig. 7). Neither the quantitation of the conversion nor the characterization of the product has been carried out.

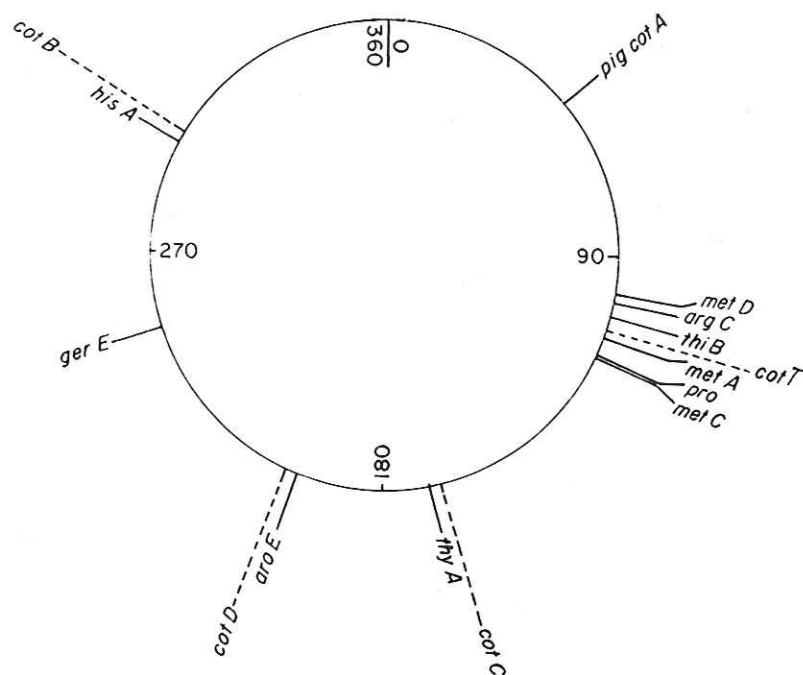


Fig. 4. Approximate location of spore coat genes (*cotA–D*, *cotT*) on the *B. subtilis* chromosome (Piggot and Hoch, 1985). Location of *cotA–D* from Donovan *et al.* (1987); for *cotT*, the data in Table 2 were used.



Table 2. Mapping of the *cotT* gene.

Recipient		% Cotransduction
Strain <sup>a</sup>	Genotype	
1A5	<i>glyB133</i>	0
	<i>metC3</i>	51
1A39	<i>argC4</i>	65
1A84	<i>metD1</i>	54
1A125	<i>metA29</i>	96
	<i>argC4</i>	82
1A652	<i>proAB</i>	39
		% Cotransformation
1A125	<i>metA29</i>	9.2
	<i>argC4</i>	1.0

a. Strain designations are from the Bacillus Genetic Stock Center. There was no cotransduction with the following markers: *cysA14*, *purA26*, *glyB133*, *ilvA1*, *pyrD1*, *thyA*, *B. gltA-292*, *aroD120*, *lys-1*, *thr5*, *hisA*, *aro-1*, *metB*, *cysC*, *purB*.

## Discussion

*cotT* is unique among the spore coat genes which have been analysed (Donovan *et al.*, 1987; Sandman *et al.*, 1988) in its amino acid composition and the apparent presence of one or more precursors. There is some evidence for *cotT* antigen in the insoluble fraction (Feng

and Aronson, 1986) perhaps in the form of di-tyrosine  $\gamma$ -isopeptide cross-linked monomers. The glycine repeats at the carboxyl terminus may be indicative of multiple chain interactions, as found in silk fibroin or collagen, and the presence of multiple peaks in HPLC (Fig. 1) is suggestive of other alterations such as, for example, components binding to this polypeptide or secondary modifications like those found in collagens (Eyre, 1980).

The evidence for precursors is based on the presence of an ORF encoding a polypeptide larger than the one isolated from spore coats, the presence of larger antigens in extracts from cells and spores, the accumulation of these larger antigens in extracts of the *GerE* mutant, and preliminary evidence for the conversion by trypsin of these larger antigens to one of 7.8 kD. In fact, the association of these potential precursors with repeatedly washed spores (roughly the same quantity as extracted from unwashed spores) implies that processing may occur on the spore surface. The inability of the *GerE* mutant to process the precursor may mean that proper coat assembly is required. The *gerE* locus appears to encode a DNA-binding protein (Holland *et al.*, 1987) and at least one other coat protein is overproduced in a *gerE* strain (Sandman *et al.*, 1988). It therefore appears to be a negative regulator of at least two spore coat genes, so overproduction of two or more coat proteins may result in altered assembly and hence the extensive pleiotropic defects in the spore coat of the *gerE* mutant (Jenkinson and Lord, 1983; Feng and Aronson, 1986).

It is also possible that one or both of the presumptive precursors *per se* is an integral part of the spore coat, although excess precursor accumulation on the spores led to altered germination rates. Extracts of wild-type spores contained little, if any, precursor (Figs 5 and 6) so if they were present, the insoluble fraction would be the most likely place.

All six of the cloned spore coat genes map at unique sites (Fig. 4) but they appear to share common upstream sequences for the binding of a unique form of RNA polymerase and perhaps for other regulatory factors such as the *gerE* protein. They are all transcribed late in sporulation, coincident with the appearance of the spore coat on the endospore, i.e. stage V of sporulation, so the apparent coordinate regulation is probably partly the result of these upstream sequences. The genes for the spore acid-soluble proteins are also unlinked but are coordinately regulated (Connors *et al.*, 1986).

Several of the spore coat proteins appear to be dispensable at least on the basis of the criteria used (i.e. heat and lysozyme resistance and germination rate of the spores) with the exception of *cotD* (Donovan *et al.*, 1987). Disruption of a more recently characterized locus, *cotE*, did result in extensive alterations of the structure of the spore coat (Sandman *et al.*, 1988). Whether or not alterations in *cotT*

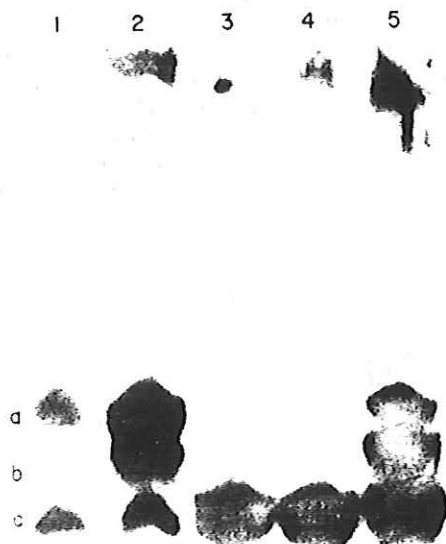


Fig. 5. Immunoblot of extracts from a late sporulating culture (greater than 60% with phase-bright endospores) or washed spores of *B. subtilis* JH642, containing the cloning vehicle pHP13 (lanes 1 and 4); cells or spores containing pT5A (lanes 2 and 5) or washed spores of *B. subtilis* JH642 (lane 3). The immunoblot was treated with rabbit antibody to the  $\text{Na}_2\text{SO}_4$  precipitate from a spore coat extract plus anti-rabbit alkaline phosphatase conjugate, as described in *Experimental procedures*. Bands labelled (a) and (b) refer to potential precursors; band (c) is the 7.8 kD spore coat protein.



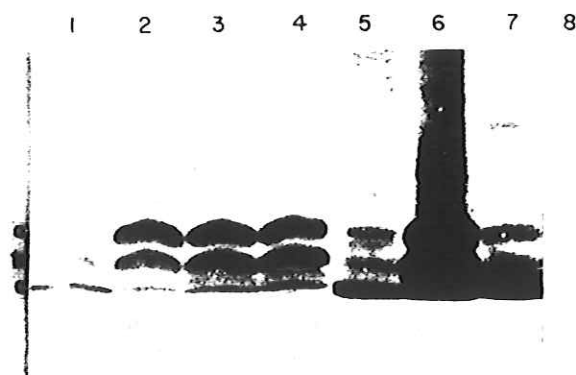


Fig. 6. Immunoblot of extracts (lanes 1–4) of sporulating cells (as in Fig. 5) or washed spores (lanes 5–7) treated as described in the legend to Fig. 5. Lanes 1 and 5: extract or spores of *B. subtilis* JH642/pHP13; lanes 2 and 6: extract or spores of *B. subtilis* JH642/pT5A; lanes 3 and 7: extract or spores of *B. subtilis* AA9-1/pHP13; lane 4: extract of *B. subtilis* AA9-1/pT5A; lane 8: as in lane 2 but treated with antibody preadsorbed with purified 7.8 kD protein bound to sepharose. Bands labelled (a) and (b) represent potential precursor antigens; band (c) represents the 7.8 kD spore coat protein.

result in phenotypic changes in spores remains to be determined.

## Experimental procedures

### Bacterial strains

The wild-type strain was *B. subtilis* JH642 (*trp*, *phe*), obtained from Dr J. Hoch. Strain AA9-1 (*gerE*, *leu*) was described by Feng and Aronson (1986). A series of strains for mapping (Dedonder *et al.*, 1977) and designated 1A3–1A10, was obtained from the Bacillus Genetic Stock Center. Strains 1A39 (*argC4*), 1A55 (*arol*, *metB5*), 1A79 (*cysC1*), 1A84 (*glyB133*, *metD*), 1A25 (*metA29*, *argC4*), 1A156 (*purB*) and 1A652 (*proAB*) were obtained from the same source.

### Isolation of a spore coat protein

Spores of *B. subtilis* JH642 were prepared from cells grown in a nutrient sporulation medium (NSM; Schaeffer *et al.*, 1963). Spore purification, preparation of coats and extraction were as previously described (Pandey and Aronson, 1979). One of the soluble spore coat proteins was salted out with  $\text{Na}_2\text{SO}_4$  (Goldman and Tipper, 1978), washed 4–5 times with distilled water, and lyophilized. The material was dissolved in 20% *n*-propanol–0.1% trifluoroacetic acid (TFA) and fractionated by HPLC on a 4.6 × 250 mm reverse phase RP-P (SynChrom Inc.) column employing a linear gradient of 20–100% *n*-propanol–0.1% TFA in a Waters system. The effluent was monitored at 230 nm and peak fractions collected manually and then lyophilized. Amino acid sequencing was done on a Model 420 gas phase sequencer and deoxyoligonucleotides were synthesized in an Applied Biosystems Model 380A DNA synthesizer.

A mixed oligonucleotide was labelled by incubation with  $\gamma$ - $^{32}\text{P}$ -ATP and polynucleotide kinase (Maniatis *et al.*, 1982) and used as a probe to screen eight pools of plasmids containing partial *Sau*3A digests of *B. subtilis* DNA (Hasnain and Thomas, 1986). The one reactive pool was then transformed into *E. coli* HB101 and colonies on filters screened with the probe. Simultaneously, *B. subtilis* JH642 DNA digested with various restriction enzymes was resolved on a 0.8% agarose gel and Southern blots prepared. A region of c. 1 kb from a *Hind*III digest reacting with the probe was electroeluted from an agarose gel and, following phenol extraction and precipitation with two volumes of ethanol, was cloned into the *Hind*III-digested shuttle vector, pLP1201 (Ostroff and Pène, 1984). Following transformation of *E. coli* HB101 and plating on L-agar plus 20  $\mu\text{g ml}^{-1}$  ampicillin, colonies were screened for tetracycline sensitivity. About 500 colonies were streaked onto Millipore HA filters (0.45  $\mu$ ) on L-agar plus ampicillin (20  $\mu\text{g ml}^{-1}$ ), lysed and screened with the probe. Both screenings yielded an identical 1.2 kb *Hind*III insert which was sequenced in both strands by the dideoxy procedure (Sanger *et al.*, 1977) after cloning into m13mp18 and m13mp19 and then deleting with exonuclease III (Henikoff, 1984). Sequences of certain regions were also confirmed by primer extension of synthetic deoxyoligonucleotides.

Since no *B. subtilis* transformants were obtained with the pLP1201 clone, the 1.2 kb *Hind*III fragment was cloned into another shuttle vector, pHP13 (Haima *et al.*, 1987). The clone containing the *Hind*III fragment in pHP13 is designated pT5A.

### Mapping

The 1.2 kb *Hind*III fragment was isolated as described above and cloned into pDE194 (Ebbole and Zalkin, 1987), an integration vehicle which replicates in *E. coli* (ampicillin resistance) but not in *B. subtilis* (expresses chloramphenicol resistance,  $\text{Cm}^R$ , when integrated). Transformants (Anagnostopoulos and Spizizen, 1961) of *B. subtilis* JH642 were selected on NSM plus 5  $\mu\text{g ml}^{-1}$  chloramphenicol. The  $\text{Cm}^R$  marker was then mapped by PBS1 transduction (Hoch *et al.*, 1967) employing the strains previously

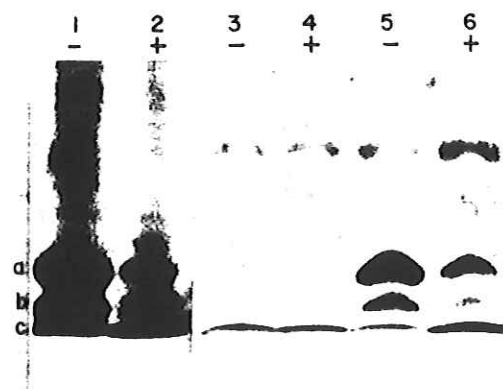


Fig. 7. Immunoblot (as in Fig. 5) of extracts of sporulating cells of *B. subtilis* JH642/pT5A (lanes 1 and 2) and washed spores of *B. subtilis* JH642/pT5A (lanes 5 and 6) or of *B. subtilis* JH642/pHP13 (lanes 3 and 4). Extracts (50  $\mu\text{g}$  of protein) were dialysed for 12 h at 4°C versus 1 l of 0.01 M Tris, pH 8.0 and then incubated at 37°C for 1 h with (+) or without (-) 1  $\mu\text{g}$  of trypsin. Bands are labelled (a), (b) and (c), as in Figs 5 and 6.

listed and subsequently by transformation. In both cases, selection was for Cm<sup>r</sup> on NSM agar plates. Various prototrophic markers were then scored on a minimal glucose medium (Spizizen, 1958) appropriately supplemented in order to determine linkage.

### RNA fractionation and S1 mapping

Total RNA was prepared from cells grown in NSM to various stages of sporulation (approximated by the appearance of cells in the phase microscope, i.e. by the percentage of dull, phase-white or bright endospores). 100 ml portions were harvested by centrifugation at 8000 rpm for 8 min in a Sorvall GSA rotor. The cells were washed twice by suspension in 20 ml each of 0.05 M Na acetate-0.1 M NaCl-1 mM EDTA, pH 5.5. A final suspension in 2 ml of this buffer was supplemented with sodium dodecyl sulphate (SDS) to 1%. After pouring of the suspension into a French pressure cell, 0.7 ml of a purified bentonite suspension (Fraenkel-Conrat *et al.*, 1961) at 32 mg ml<sup>-1</sup> was added. The cells were extruded at 9000 psi directly into an equal volume of phenol saturated with 0.03 M Tris, pH 7.6. Following extraction, the aqueous phase was precipitated with two volumes of ethanol and some DNA was removed by winding onto a sterile glass rod. After incubation at -20°C for 20 min, the precipitate was collected and dissolved in 1 ml 0.01 M Tris-2 mM MgCl<sub>2</sub>, pH 7.6, and ribonuclease-free deoxyribonuclease I (BRL) was added to 5 µg ml<sup>-1</sup>. Following incubation at 37°C for 40 min, the phenol extraction was repeated twice and the RNA collected by ethanol precipitation (after addition of Na acetate to 0.3 M). The final precipitate was dried, dissolved in distilled water and the concentration determined by measuring the adsorption at 260, 280 and 320 nm. Portions were frozen in sterile tubes at -70°C.

The RNA was fractionated for Northern blots as described by Thomas (1980). S1 nuclease mapping (Burke, 1984) was done by labelling either an 898 bp *Hind*III-*Hha*I fragment of the insert with polynucleotide kinase plus γ-[P<sup>32</sup>]-ATP or by oligonucleotide primer extension (dashed overlined region, GATGCTTAAGTTGGGTGTATG in Fig. 2) employing the 1.2 kb *Hind*III fragment as template. Klenow fragment and α-[P<sup>32</sup>]-dCTP (Maniatis *et al.*, 1982).

### Immunoblotting

For immunoblotting (Towbin *et al.*, 1979) extracts of cells grown in NSM (plus or minus 5 µg ml<sup>-1</sup> chloramphenicol) were prepared by centrifuging 10–30 ml portions in a Sorvall SS-34 rotor at 8000 rpm for 10 min. The pellets were washed once in 5 ml 0.03 M Tris, pH 7.6, suspended in 50 µl Tris plus 40 µg lysozyme, and incubated at 37°C for 10 min. After addition of SDS to 1.0%, and phenyl methyl sulfonyl fluoride (PMSF) to 5 mM, the suspensions were placed in a boiling water bath for 3 min. The tubes were cooled, centrifuged in an Eppendorf for 5 min and the supernatants collected. The pellets were suspended in 30 µl UDS (Sastri *et al.*, 1983) plus 5 mM PMSF and incubated at 37°C for 30 min. Following centrifugation in an Eppendorf for 7–8 min, the supernatants were pooled and portions removed for Folin protein determinations. Equal quantities of protein in the extracts were fractionated on 20% SDS-PAGE. After transfer to nitrocellulose, treatment was with rabbit antibody to the Na<sub>2</sub>SO<sub>4</sub> precipitate of a

spore coat extract plus anti-rabbit alkaline phosphatase conjugate (Promega). Control treatments included pre-immune serum and antibody absorbed with the HPLC purified protein conjugated to cyanogen bromide activated Sepharose 4B (Sigma).

Spores were harvested after 24–36 h incubation by centrifugation in a Sorvall SS34 rotor at 12000 × g for 10 min. They were washed with 10 ml portions of 1 M KCl and distilled water (twice), suspended in 10 ml 0.03 M Tris-HCl, pH 7.6 plus 50 µg ml<sup>-1</sup> lysozyme, and incubated at 37°C for 1 h. They were then washed with 1 M KCl, distilled water, 0.05% SDS, and again with distilled water (three times). Intact spores were counted in a Petroff-Hauser chamber and then extracted as described by Donovan *et al.* (1987).

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## PROPERTIES OF *BACILLUS SUBTILIS* SPORES WITH ALTERATIONS IN SPORE COAT STRUCTURE

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### I. INTRODUCTION

The proteinaceous coat of *Bacillus* spores is believed to be important in both resistance to adverse environmental conditions and in the ability to respond to germinants (Aronson and Fitz-James, 1976; Dion and Mandelstam, 1980). The composition of this structure differs considerably among members of the genus. In *Bacillus subtilis* it consists of a group of 10-20 extractable polypeptides which may vary in size from 7 kd to 65 kd (Goldman and Tipper, 1978; Pandey and Aronson, 1979; Jenkinson *et al.*, 1981), plus an insoluble proteinaceous fraction which may comprise up to 30% of the total spore coat protein (Pandey and Aronson, 1979). In contrast, the spore coats of some other members of the genus such as *Bacillus cereus* and *Bacillus megaterium* contain only a few major low molecular weight species (Aronson and Fitz-James, 1976; Stewart and Ellar, 1982).

The reason for the heterogeneity of the *B. subtilis* spore coat polypeptides and the mechanism by which they are assembled into the finished structure are unknown. As a preliminary step in the elucidation of their functions, individual spore coat genes have been cloned and analyzed (Donovan *et al.*, 1987; Zheng *et al.*, 1988; Aronson *et al.*, 1989). Many of the polypeptides are small, with unusual compositions which frequently include regions of repeating amino acid sequences. So far, only the absence of the CotE protein (among the 5-6 deleted) has resulted in extensive alteration of the coat structure (Zheng *et al.*, 1988).

CotT is of interest because it is a prevalent low molecular weight spore coat polypeptide which may also be a component of the coat insoluble fraction (Feng and Aronson, 1986). It is also the only coat protein identified which is processed from a precursor (Aronson *et al.*, 1989).

The transcription and translation of the *cotT* gene have been further investigated and are described here. In addition the effects of extra copies and deletions of this gene and one encoding another low molecular weight protein, CotD (Donovan *et al.*, 1987), on spore coat structure, resistance properties of the spore and the ability of spores to respond to a variety of germinants have been examined. There are effects of overexpression on spore germination and a specific germination defect due to the absence of the CotT protein.

## II. TRANSCRIPTION AND TRANSLATION OF THE *cotT* GENE

The sequence of the complete open reading frame of the *cotT* gene is shown in Fig. 1. The original transcription start site (Aronson *et al.*, 1989) as indicated by S1 nuclease mapping (Burke, 1984) is shown by the thin arrow at nt 593. A second major transcription start site was established by reverse transcriptase mapping (Wu *et al.*, 1989) and is shown by the large open arrow at nt 505. Possible sites for the binding of RNA polymerase are represented by the broken overline while the putative ribosome binding site is underlined. The *leu* residue shown in bold face is the predicted translation start site. The large solid arrow represents the beginning of the sequenced CotT protein extracted from spores (*i.e.* NH<sub>2</sub> Gln-Pro-Tyr, etc.).

Evidence to support translation initiation at the *leu* residue was provided by studies with strain *B. subtilis* 642/T5A (Aronson *et al.*, 1989) which carries the *cotT* gene on the low copy number vector pHP13 (Haima *et al.*, 1987). There were two presumptive precursors present in immunoblots of extracts from late sporulating cultures (Aronson *et al.*, 1989) and in spore coat extracts (unpublished results). The larger was electroblotted onto a polyvinylidene difluoride membrane (Matsudaira, 1987), excised and subjected to protein sequencing. The amino terminal region matched that predicted from the *cotT* DNA sequence starting at the TTG encoded *leu* residue. The predicted size of this precursor is 10.1 kd, but its electrophoretic mobility is similar to that of lysozyme (14.4 kd) possibly due to its unusual amino acid composition.

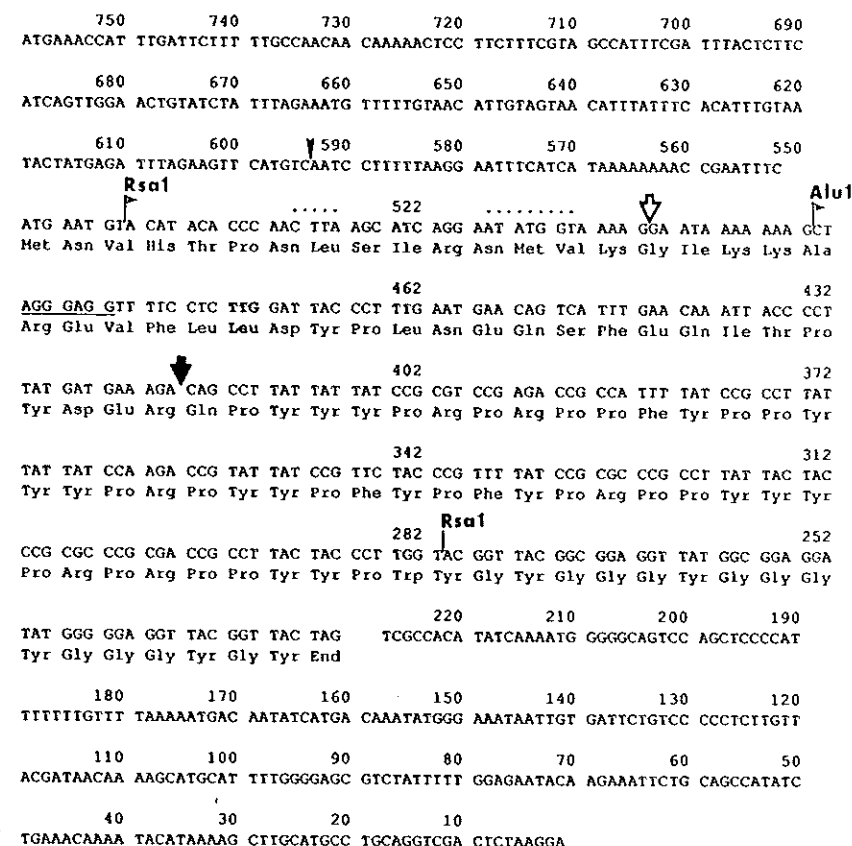


Figure 1. Nucleotide sequence of the open reading frame of the *cotT* gene as modified from Aronson *et al.* (1989). See text for details.

Further evidence that the precursor is processed at the *arg-gln* site (shown by the thick arrow in Fig. 1) to produce the 7.8 kd mature spore coat protein was provided by site-directed mutagenesis (Kunkel, 1985) of R191. The altered gene was integrated into the chromosome of *B. subtilis* 642 creating a partial diploid (*B. subtilis* 642/PD633) with one wild-type and one altered gene copy. The large CotT precursor accumulated as determined by immunoblots and SDS/PAGE of stained gels of spore extracts (unpublished results). This accumulation of precursor was not found in a strain containing two wild-type *cotT* genes (see Section IV).

### III. CONSTRUCTION AND PROPERTIES OF *cotT* DELETIONS

The *AluI*-*RsaI* fragment (nt 280-493) shown in Fig. 1 was cloned into the integrative vector pDE194 (Ebole and Zalkin, 1987) and introduced into *B. subtilis* 642 by transformation. Recombination within this cloned region and the chromosomal *cotT* gene resulted in integration of the vector into the chromosome and production of two incomplete gene copies (Fig. 2). The first of these contains the promoter region (A) and part of the protein coding region, but lacks the carboxyl portion of the protein (B). The second copy, although intact, lacks the promoter region (A). This construct (NB200) was confirmed by Southern hybridization (Southern, 1975) and there was no detectable CotT protein in SDS/PAGE of spore coat extracts, nor in immunoblots of extracts from spores or late sporulating cells (unpublished results). Electron micrographs of spore sections of strain NB200 differed from the wild-type in that the outer spore coat was less electron-dense than the comparable layer from wild-type spores (P. C. Fitz-James, personal communication). Despite these differences, NB200 spores were as resistant to heat, lysozyme and octanol as wild-type spores. Similarly their response to the germinants L-alanine (plus 1 mM inosine) at a variety of concentrations between 0.1 mM and 10 mM and to Penassay broth were the same as the wild-type. NB200 spores responded slowly, however, to a germination mixture consisting of 0.1 M KCl, 5.6 mM glucose, 5.6 mM fructose and 3.3 mM L-asparagine (Wax and Freese, 1968; Fig. 3). There was no change in the germination of NB200 or the wild-type spores by lowering the glucose or fructose concentration tenfold.

Another *cotT* deletion was constructed by integration of the 263 bp *RsaI* gene fragment (nt 280-543 in Fig. 1) as in Fig. 2. The resulting strain (NB2) contained one complete copy of *cotT* including the presumptive promoter but little if any CotT protein was found in spore coat extracts, or in extracts from sporulating cells. Perhaps the intact gene copy lacks upstream regions important for *cotT* transcription. As anticipated, the germination of NB2 spores like those of NB200 was slower than the wild-type but only in the glucose, fructose and L-asparagine mixture.

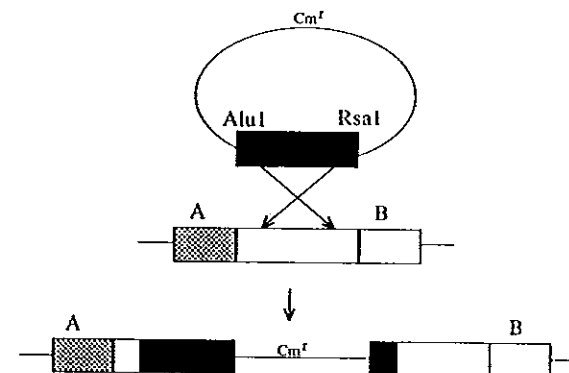


Figure 2. Construction of the *cotT* deletion strain NB200 by integration into the chromosomal gene copy of a vector containing an internal *cotT* *AluI*-*RsaI* fragment. A is the promoter region and B is the portion of the gene encoding the carboxyl end of the CotT protein.

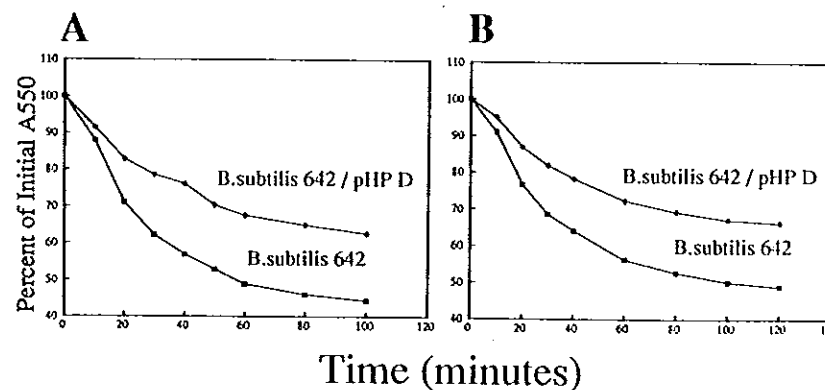


Figure 3. Germination of *B. subtilis* 642 and NB200 spores in 0.05 M Tris pH 7.6 containing 0.1 M KCl and 5.6 mM glucose, 5.6 mM fructose and 3.3 mM L-asparagine at 37°C both without (A) and with (B) heat activation at 80°C for 20 min.

#### IV. OVERPRODUCTION OF CotT

Spores of *B. subtilis* 642/T5A in which the *cotT* gene is present on the vector pHP13 at 5-6 copies per genome responded slowly to L-alanine plus inosine (Fig. 4A), the glucose, fructose and L-asparagine mixture (Fig. 4B) as well as Penassay broth (as in Fig. 4A) even when heat activated prior to germination. As previously described (Aronson *et al.*, 1989), extra CotT precursor proteins can be extracted from these spores and they appear to have a thickened inner spore coat (P. C. Fitz-James, personal communication), so altered spore coat structure and composition could account for the germination defect.

*B. subtilis* 642/PD1 was constructed by integration of the entire wild-type *cotT* gene into the chromosome resulting in a strain with two functional chromosomal gene copies (confirmed by Southern hybridization and increased *cotT* mRNA content). Spores of this strain germinated as well as the wild-type in response to all of the germinants tested and there was no excessive accumulation of CotT precursors in immunoblots or in SDS/PAGE of protein extracts from purified spore coats (unpublished results). Apparently, more than two gene copies are needed for the excessive accumulation of CotT protein in the spore coat with the resulting germination defect.

#### V. STUDIES WITH *cotD* MUTANTS

The clone of *cotD* and a deletion strain (BZ109; Donovan *et al.*, 1987) were generously provided by B. Zheng and R. Losick. When the intact gene was transferred to the vector pHP13 and transformed into *B. subtilis* 642, there was an increased amount of protein of the size of CotD in SDS/PAGE of spore extracts (unpublished results). Germination of this overproducing strain was slower than the wild-type in L-alanine plus inosine (Fig. 5A), the glucose, fructose and L-asparagine mixture (Fig. 5B) and Penassay broth (as in Fig. 5A) with or without heat activation but in no case was the impairment as great as for the CotT-overproducing strain (Fig. 4). Strain BZ109 was constructed by the insertional inactivation of the *cotD* gene (Donovan *et al.*, 1987) but in contrast to the *cotT* deletion (Fig. 3) there was no discernible effect on germination.

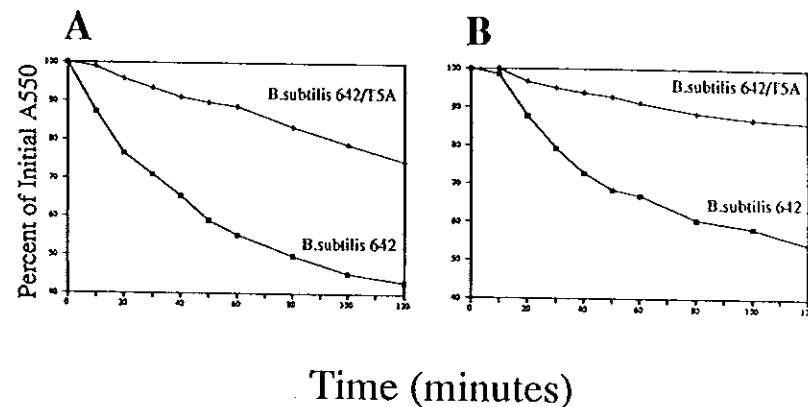


Figure 4. Germination of *B. subtilis* 642 and *B. subtilis* 642/T5A in 0.05 M Tris pH 7.6 containing 0.1 M KCl and (A) 1 mM inosine plus 1 mM L-alanine, or, (B) 5.6 mM glucose, 5.6 mM fructose and 3.3 mM L-asparagine. Spores were heat activated at 80°C for 20 min.

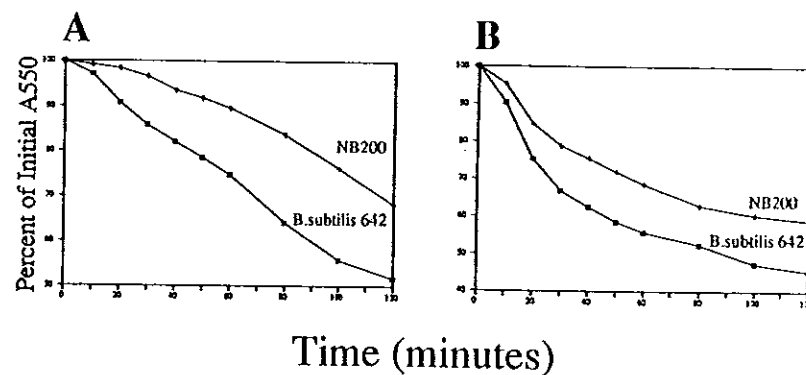


Figure 5. Germination of *B. subtilis* 642 and *B. subtilis* 642/pHP D spores as in Figures 4A and 4B, but without heat activation.



## VI. SUMMARY

A major transcription initiation site for the *cotT* gene has been determined. The predicted translation product has an N-terminal *leu* and a molecular weight of 10.1 kd, whereas the mature spore coat protein is 7.8 kd. Processing is at the *arg-gln* bond and an R191 mutation resulted in accumulation of CotT precursor protein indicating that a trypsin-like protease may be involved. There was no effect on spore properties or germination in the *cotT* heterozygote but the effect of this recessive mutation by itself must be determined.

The amino acid sequence of the amino terminal portion of the larger CotT precursor protein is in agreement with the predicted amino acid sequence from the *cotT* gene. In addition to the 10.1 kd precursor there may be a second, somewhat smaller precursor in sporulating cells and in spore coats of the strain which over produces CotT protein.

Deletion of the *cotT* gene resulted in a thinning of the outer spore coat and an impaired germination response, but only to a mixture containing glucose, fructose and L-asparagine. Altering the concentration of glucose or fructose did not affect the germination rate implying that the CotT protein is not directly involved. This protein is probably not a receptor for specific germinants but more likely, its function in spore coat structure is important for the accessibility of this particular germination mixture to its site of action.

Another *cotT* deletion strain lacked only a region upstream of the promoter but there was no CotT antigen synthesis implying that a transcriptional regulatory region is within the deleted portion.

Germination of a *cotT* diploid strain did not differ from the wild-type in any system tested. Overexpression of the gene on a low copy number vector at 5-6 copies per genome however, resulted in spores which were slow to respond to all germinants. This inhibition probably results from the deposition of excess CotT precursor, perhaps in the inner spore coat as is suggested by electronmicrographs. The correct stoichiometry of coat proteins is probably important for normal coat assembly and thus germination.

Response of a mutant lacking another coat protein (CotD) was indistinguishable from the wild-type for all germinants tested. Excess synthesis of this protein resulted in slower germination than the wild-type but not to the same extent as was caused by CotT protein production.

## ACKNOWLEDGEMENTS

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